CERTIFICATE OF MAILING
37 C.F.R. §1.8

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9/3/04
Date

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Shuyuan Zhang, Capucine Thwin, Zheng Wu, Toohyon Cho, Shawn Gallagher

Serial No.: 09/203,078

Filed: December 1, 1998

For: METHOD FOR THE PRODUCTION AND PURIFICATION OF ADENOVIRAL VECTORS

Group Art Unit: 1645

Examiner: Shanon A Foley

Atty. Dkt. No.: INRP:081

SECOND DECLARATION OF SHUYUAN ZHANG UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

I, Shuyuan Zhang, do declare that:

1. I am a inventor of the claims in the above-referenced application.

2. I am also an inventor of U.S. Patent 6,194,191 ("the '191 patent"), which I understand to be the same disclosure as WO 98/22588 ("PCT application") (collectively "Zhang disclosures").

3. I understand claims 1-32, 38-49, and 51-62 in the above-referenced application have been rejected as anticipated or obvious in the Office Action dated June 3, 2004 ("Action")

based on the Zhang disclosures. Claims 1 and 47 in the rejected set of claims are independent claims, and the remaining claims are dependent from either claim 1 or claim 47.

4. I understand that the Action asserts that the Zhang disclosures anticipate "a process of preparing adenovirus by preparing a culture of producer cells essentially homogenous with respect to growth, infecting the producer cells with adenovirus between mid-log and stationary phase, and harvesting the adenovirus and placing the virus into a pharmaceutically acceptable composition." Action at page 9.

5. This process was invented by me and the other inventors common to the above-referenced patent application and the inventors listed on the cited Zhang disclosures.

6. Furthermore, in studies conducted at Introgen Therapeutics involving the growth of 293 cells in T-150 flasks, a cell doubling time of approximately 30 hours during exponential growth phase was observed. See pages 9 and 12 of Appendix A (Quality Assurance Report).

7. I hereby declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the referenced patent application or any patent issued thereon.

02 Sep 04

Date

Shuyuan Zhang, Ph.D

INTROGEN
Therapeutics, Inc.

Quality Assurance Report
QR04013

Characterization of 293 Working Cell Bank (CN001263)

Author:

Hai Pham **Process Development Assistant**	**Date**

Approval:

	Date

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Purposes

The purpose of this study is to characterize the 293 Working Cell Bank C/N001263 with regard to the cell thaw viability and subsequent cell growth doubling time and cell-specific virus productivity.

Responsibility

This study was performed by Process Development personnel in the Process Development lab at Introgen.

Materials

Cells: 293 Working Cell Bank, P/N 08-00005, C/N 001263
Media: P/N 01-00394, C/N 003186
T-150 flasks: P/N-06-00040, CN-003406
rProtease: P/N-01-00476, CN-003335
Trypan blue: PN-T-8154, Lot-88H2322

Study Plan and Procedures

The cell thaw characteristics and the subsequent cell growth of 293 WCB P/N 08-00005, C/N 001263 were studied. The cell doubling time at the exponential growth phase was determined in the second passage after thaw. Cell specific Ad5CMV-p53 productivity was determined at the later lag growth phase. The study results will be used to characterize the 293 WCB and to qualify the WCB for production use.

1. Cell thaw

    1.1. Request 5 vials of 293 Working Cell Bank from Materials Management. The 5 vials of cells should be retrieved from different locations in the liquid nitrogen storage tank.
    1.2. Thaw the cells inside the PD incubator set at 37°C for < 15 minutes till the vials are visibly thawed.
    1.3. Transfer the cells from each of the 5 vials into 5 T-150 flasks pre-filled with 99mL of warmed media.
    1.4. Mix the cells inside the media. After mixing, take 1mL of sample from each flask. Stain the cells with trypan blue at 1:1 ratio and perform a cell count to determine the cell viability and cell concentration per LT004-01.
    1.5. Seed one half of the cell suspension from each T-150 flasks into 2 T-150 flasks (mimic the manufacturing procedures per BR089-10) for a total of 10 T-150 flasks. Add more media to each flask to a final volume of 50mL. Place the flasks inside a CO_2 incubator set at 37°C.
    Note: The cell seeding procedure has since been changed to a specific cell seeding density of $3.5x10^4$ cells/cm^2 in BR089-11 for better control.

1.6. Allow the cells to grow to approximately 90% confluence. Observe the flasks daily and document the cell confluence.

2. Cell doubling time and virus productivity

2.1. When cell confluence reaches approximately 90%, detach the cells from the growth surface using 3mL of rProtease after aspirating the spent media from each flask. After 2-8 minutes, inactivate the rProtease by adding 17mL of media to each flask.
2.2. Pipetting the cell suspension and take a 1 mL sample to perform a cell count.
2.3. Use the cells to seed 15x T-105 at a seeding density of $2.5x10^4$ cells/cm^2. Add media to a final volume of 50mL. Place the flasks inside the CO_2 incubator set at 37°C.
2.4. On day 2 post cell seeding, harvest 2 -150s for cell growth determination.
2.5. On day 3 post cell seeding, harvest another 2 -150s for cell growth determination.
2.6. On day 4 post cell seeding, harvest another 2 -150s for cell growth determination.
2.7. On day 5 post cell seeding, harvest another 2 -150s for cell growth determination.
2.8. On day 5 post cell seeding, infect 2 T-105s with Ad5CMV-p53 virus for virus productivity determination. Place the infected T-150 flasks inside a CO_2 incubator set at 35°C.
2.9. On day 6 post cell seeding, harvest 2 -150s for cell growth determination.
2.10. On day 7 post cell seeding, harvest 2 -150s for cell growth determination.
2.11. Calculate the cell doubling times on individual date to determine the cell doubling time at the logarithmic growth phase.

3. HPLC analysis

3.1. On day 6 post virus infection, harvest the culture media.
3.2. Treat the virus harvest with Benzonase at 100unit/mL for 1 hour at 37°C.
3.3. Filter the treated harvest using a 0.2um filter.
3.4. Analyzed on the PD HPLC for virus titer determination using the Resource Q method.

Results and Discussion

Cell growth characterization (see lab notebook 207 page 7)

1. Initial cell growth after thaw

Different vials of cells were retrieved from the liquid nitrogen storage tank by Materials Management. The vial locations are shown below.

Table 1 Location of vials thawed

Vial	Liquid nitrogen storage tank location
1	C-7

Vial	Liquid nitrogen storage tank location
2	C-8
3	C-9
4	C-10
5	C-11

The cells were thawed inside the PD incubator individually. Thaw time was approximately 9 minutes. Thawed cells were transferred into 99mL of media inside a T-150 flask. Cell concentration and viability were determined after trypan blue staining. The data is shown below.

Table 2 Cell viability and total viable cells after thaw

Vial	Viability (%)	Viable cell conc. (/mL)	Total viable cells
1	85	$1.0x10^5$	$1.0 x10^7$
2	91	$1.7x10^5$	$1.7 x10^7$
3	81	$1.5 x10^5$	$1.5 x10^7$
4	85	$1.6 x10^5$	$1.6 x10^7$
5	74	$9.2 x10^4$	$9.2 x10^6$

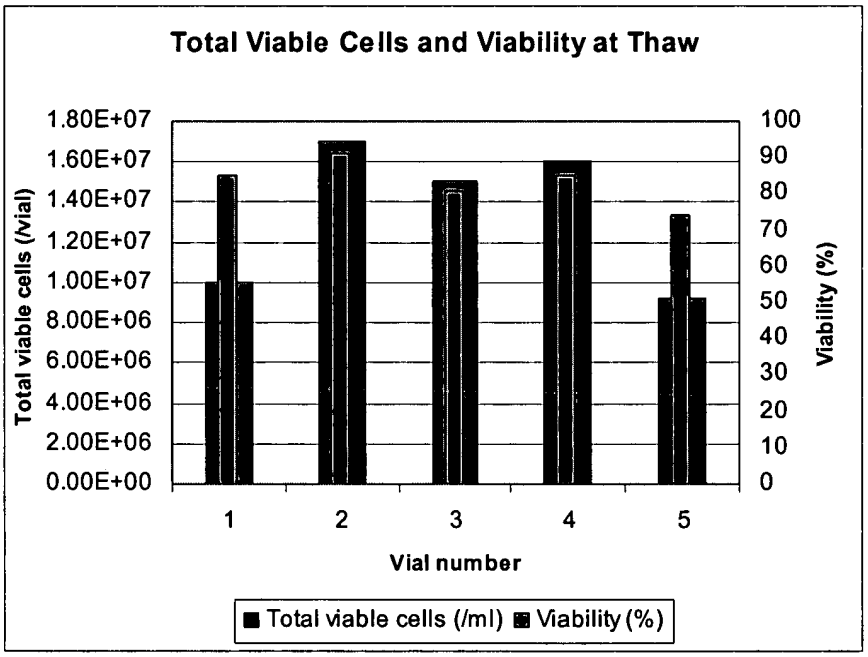


Figure 1 Cell viability and total viable cells after thaw

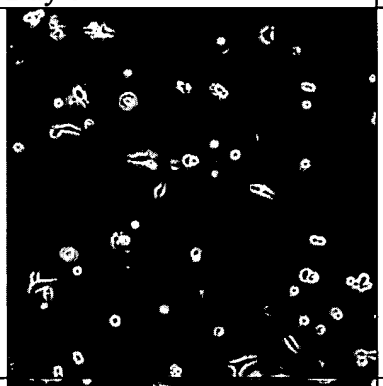
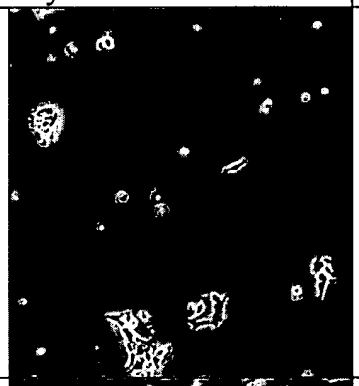
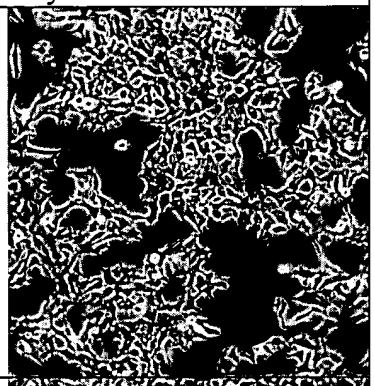
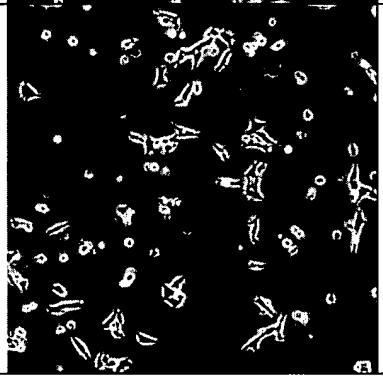
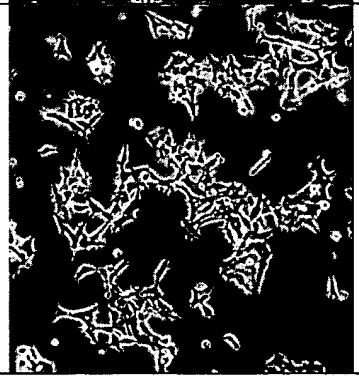
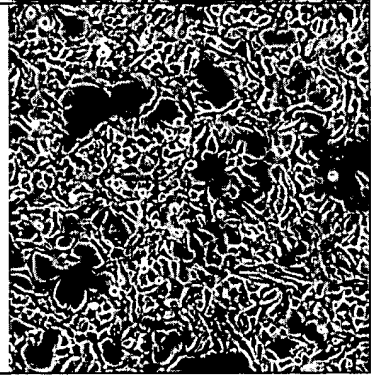
Variation in total viable cells and viability were seen among the 5 vials thawed. The variations are shown below.

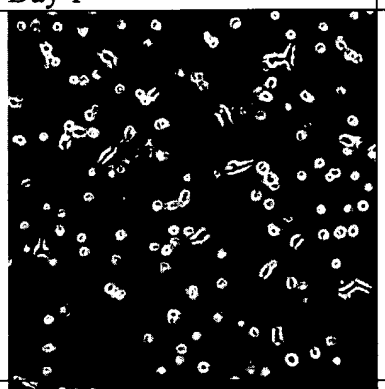
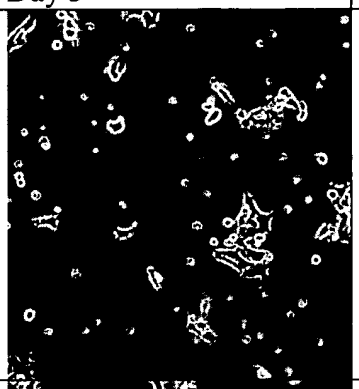
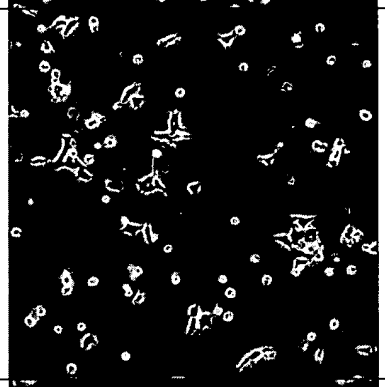
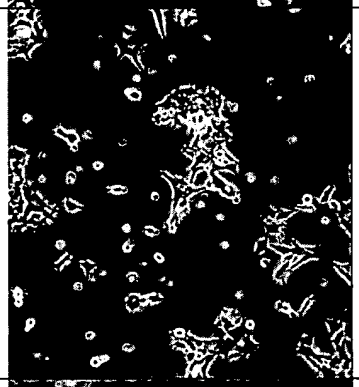
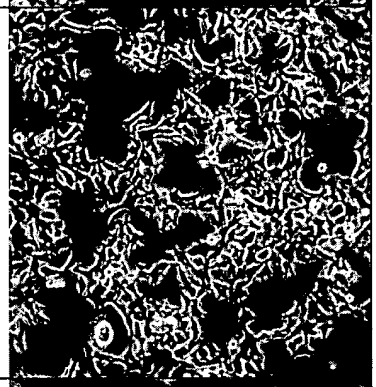
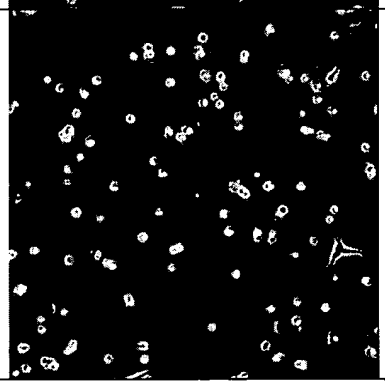
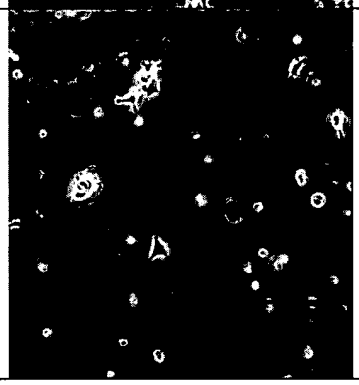
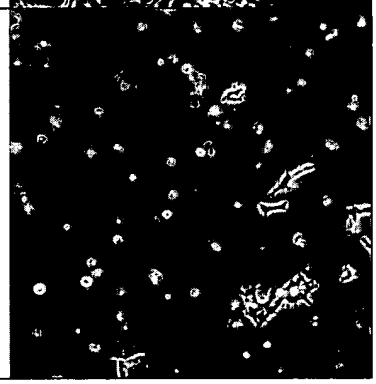
Table 3 Variations of cell viability and total viable cells in vials

	Total viable cells (/vial)	Viability (%)
Average	$1.3x10^7$	83
Standard deviation	$3.6x10^6$	6.3

25mL (one quarter of the total viable cells) of the thawed cell suspension were used to seed a T-150 flask with a total of 50mL of culture media. The cells were incubated inside the PD CO_2 incubator at 37°C. Pictures of cell confluence and morphology were taken on day 1, 3, and 5 post cell seeding.

Table 4 Pictures of cell growth

Vial	Day 1	Day 3	Day 5
1			
2			

Vial	Day 1	Day 3	Day 5
3			
4			
5			

Unexpectedly, minimal cell growth was seen for cells recovered from vial #5 after 5 days of culture. Cells from vial #5 had the lowest viability and least amount of total viable cells. The cell seeding density for vial #5 was $1.5x10^4$ cells/cm^2, which is lower than the historical cell seeding density of $\geq 2.5x10^4$ cells/cm^2. The low cell seeding density and the relatively low cell viability from vial #5 is expected to have caused the lack of cell growth. More vials stored in the C-11 location, which vial #5 was retrieved from, will be tested to confirm the low cell density and viability. Study results will be attached to this QR as an addendum. However, vials in other locations can be used for manufacturing use.

After 7 days of growth, cells were harvested from the T-150 flasks using rProtease. The amount of cells harvested from each set of flasks is shown below.

Table 5 Cell generations and doubling time

Vial	Viability (%)	Cell density (cells/cm^2)	Total viable cells (/T-150 flask)	Cell generations	Average cell doubling time (hours)
1	97	$1.8x10^5$	$2.7x10^7$	3.4	49
2	99	$2.2x10^5$	$3.3x10^7$	3.0	56
3	98	$1.2x10^5$	$1.8x10^7$	2.3	73
4	98	$2.0x10^5$	$3.0x10^7$	2.9	58
5	98	$1.2x10^4$	$1.8x10^6$	No growth	N/A

Cells recovered from vials 1-4 grew satisfactorily with average cell doubling times in the range of 49-73 hours. Consistent with the cell confluence picture shown above, no cell growth was seen for vial #5 after 7 days of culture. To prevent future lack of cell growth from thaw, 2 vials of the WCB are recommended to be thawed. Furthermore, to prevent the variation in cell seeding density caused by variation in total number of viable cells from each vial, a target cell seeding density of $3.5x10^4$ cells/cm^2 is recommended for cell seeding after thaw.

2. Second cell passage after thaw and cell doubling time

Cells harvested on day 7 post thaw were used to seed 15x T-105 at a seeding density of $2.5x10^4$ cells/cm^2. From day 2 post cell seeding, 2x T-150 flasks were harvested for cell growth determination. Cell growth data is shown below.

Table 6 Growth characteristics of the 293 WCB P/N 08-00005, C/N001263

Days of growth	Cell density (flask 1/2, /cm2)	Average cell density (/cm2)	Cell viability (flask 1/2, %)	Average cell viability (%)	Cell doubling time (hr)
0	$2.5x10^4$	$2.5x10^4$	97	97	N/A
2	$5.9x10^4/6.4x10^4$	$6.2x10^4$	97/98	98	37
3	$1.1x10^5/1.0x10^5$	$1.1x10^5$	97/98	98	29
4	$2.0x10^5/1.8x10^5$	$1.9x10^5$	97/98	98	30
5	$2.5x10^5/2.6x10^5$	$2.6x10^5$	97/97	97	53
6	$4.0x10^5/3.8x10^5$	$3.9x10^5$	97/98	98	41
7	$4.3x10^5/4.5x10^5$	$4.4x10^5$	97/96	97	136

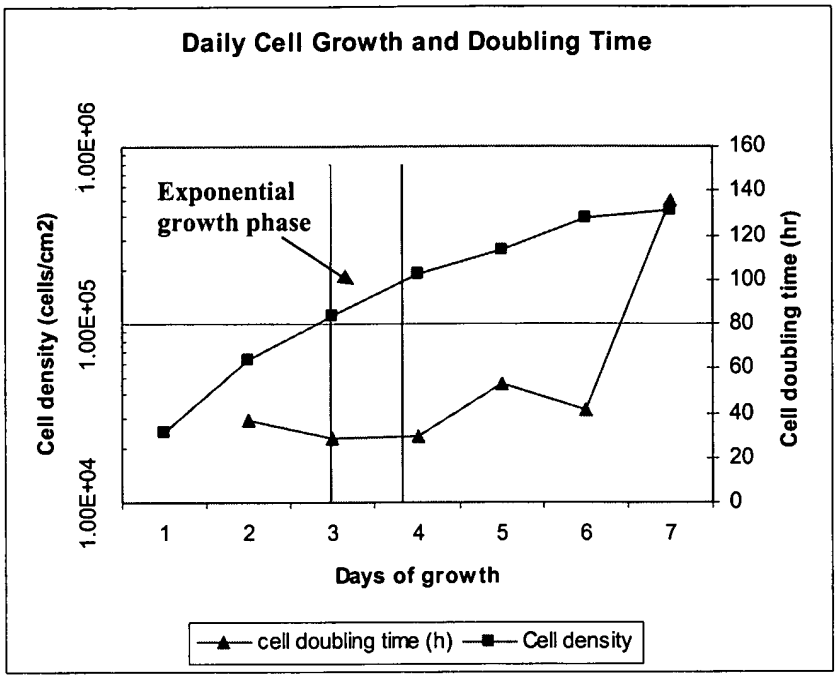


Figure 2 Characteristic growth curve of the 293 WCB P/N 08-00005, C/N001263

Cell grew exponentially on day 3 and 4 in culture. The characteristic exponential cell doubling time is approximately 30 hours. Cells appeared to be over confluent and went into lag phase after 6 days of culture. For optimal cell growth and expansion purpose, cells should be harvested on day 5 or day 6 post seeding at a density of $2.5x10^4$ cells/cm^2. This is consistent with the results documented in **QR02069** *Growth Curve Study of INT293 Cells* and the cell inoculum development procedure used in the ADVEXIN Manufacturing process.

Virus productivity characterization (see lab notebook 207 page 7)

On day 5 post cell seeding, 2 T-105 flasks were infected with Ad5CMV-p53 virus at a multiplicity of infection (MOI) of 100 vp/cell for virus productivity determination. The detailed procedures for virus infection and HPLC analysis are shown above in the **Study Plan and Procedures.** Virus productivity data as analyzed by PD HPLC is shown below.

Table 7 Ad5CMV-p53 virus productivity

Virus productivity (flask 1/2, vp/cell)	Average virus productivity (vp/cell)	Virus yield/T-150 flask (vp)
89000/86000	88000	$3.4x10^2$

The virus productivity is comparable to the historical virus productivity from 293 WCBs obtained in PD (see **QR04017** *ADVEXIN End of Production Cell Evaluation (C/N05498001/C/N001263)*).

Comparison of cell thaw using incubator and 37°C water bath (see lab notebook 207 page 13)

To examine the impact of cell thaw time on the recovery of cells from liquid nitrogen storage, 2 vials of WCB (C/N 001263) were thawed inside the PD incubator, while another 2 vials were thawed inside a 37°C water bath. Thawed cells were transferred into 99mL of media inside a T-75 flask. Cell concentration and viability were determined after trypan blue staining. The data is shown below.

Table 8 Comparison of incubator and water bath thaw of cells

Vial	Method of thaw (thaw time)	Liquid nitrogen storage tank location	Viability (%)	Viable cell conc. (/mL)	Total viable cells
1	Incubator (9 min)	C-7	85	$1.4x10^5$	$1.4 x10^7$
2	Water bath (2 min, 15 sec)	C-8	95	$1.7 x10^5$	$1.7 x10^7$
3	Incubator (9 min)	C-9	83	$1.5 x10^5$	$1.5 x10^7$
4	Water bath (2 min, 20 sec)	C-10	94	$1.8 x10^5$	$1.8 x10^7$

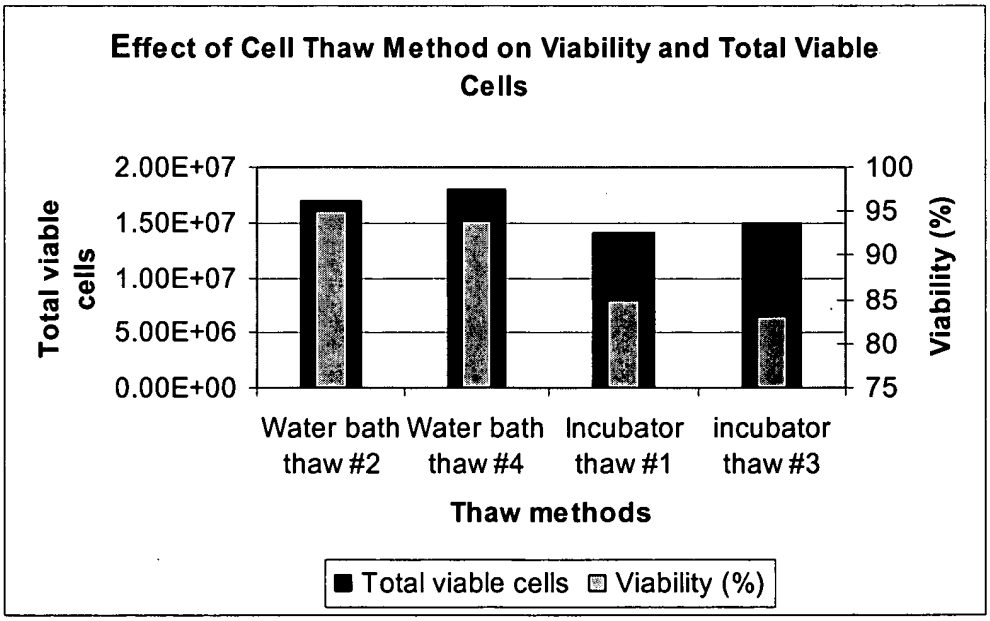
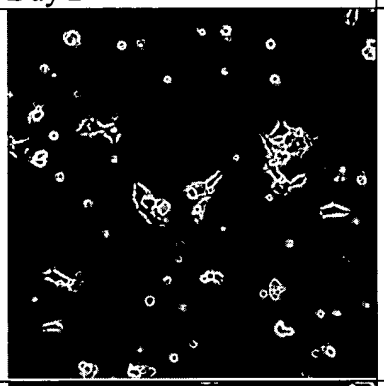
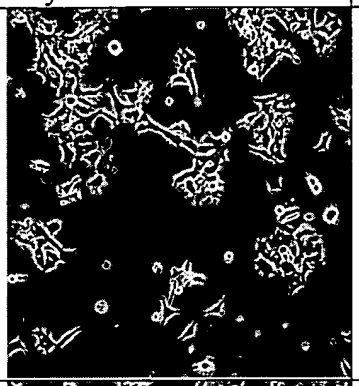
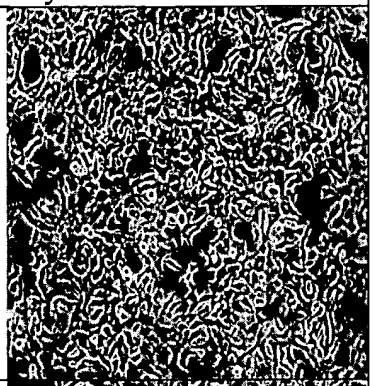
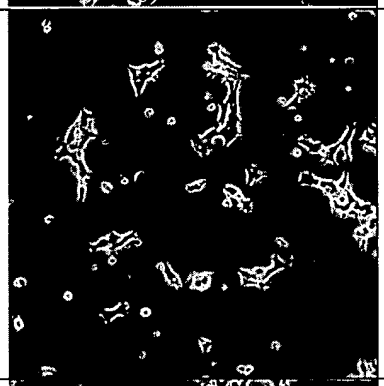
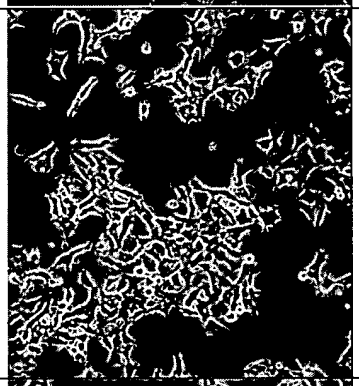
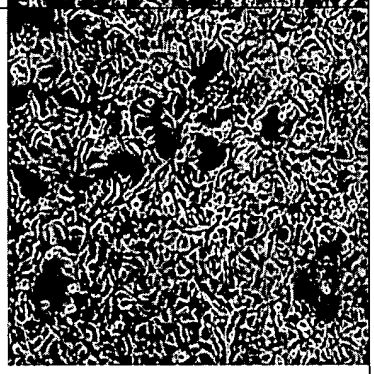
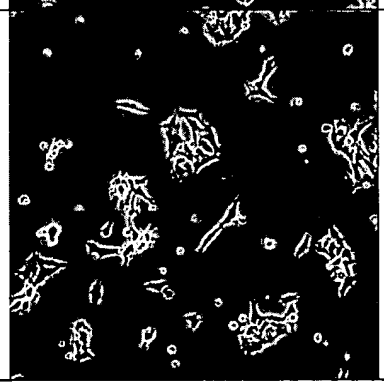
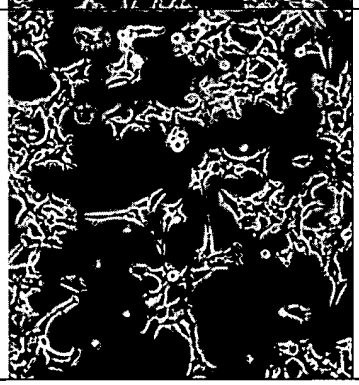
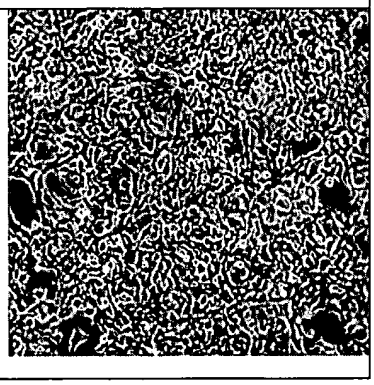
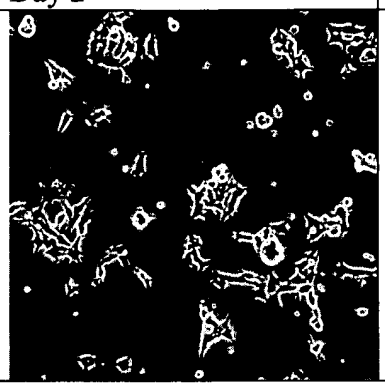
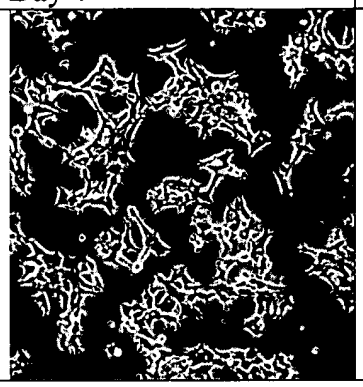
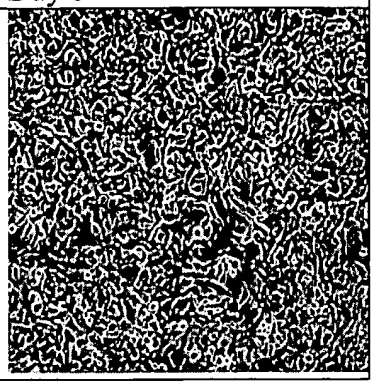


Figure 3 Comparison of incubator and water bath thaw of cells

Better initial cell viability was seen when thawed inside the 37°C water bath. Half of the thawed cells from each vial were seeded equally into 2x T-150 flasks and the cells were allowed to grow inside the CO_2 incubator. Pictures of cell confluence and morphology were taken on day 2, 4, and 6 post cell seeding.

Table 9 Pictures of cell growth

Vial	Day 2	Day 4	Day 6
1			
2			
3			

Vial	Day 2	Day 4	Day 6
4			

Despite better initial cell viability was seen when thawed inside the 37°C water bath, no apparent difference in cell attachment and growth was observed between cells thawed inside the incubator versus thawed inside the 37°C water bath. Cells were harvested on day 6 for cell counting purpose. The cell growth data is shown below.

Table 10 Comparison of cell growth thawed by the incubator and water bath methods

Vial	Viability (%)	Cell density at harvest (cells/cm^2)	Cell density at seeding (cells/cm^2)	Cell generations	Average cell doubling time (hours)
1 (Incubator thaw)	96	$1.1x10^5$	$2.4x10^4$	2.2	65
2 (Water bath thaw)	97	$1.6x10^5$	$2.8x10^4$	2.5	58
3 (Incubator thaw)	97	$1.7x10^5$	$2.5x10^4$	2.8	51
4 (Water bath thaw)	98	$1.7x10^5$	$2.9x10^4$	2.6	55

Similar to the microscopic observation of cell growth, no significant difference in cell growth was seen according to the cell generation data. Therefore, for simplicity and convenience purpose, it is recommended thawing the WCB vials inside the incubator.

Conclusions

The cell thaw viability, cell doubling time in the exponential growth phase and cell-specific Ad5CMV-p53 virus productivity were determined for the 293 WCB P/N 08-00005, C/N001263. Out of 9 vials recovered from liquid nitrogen storage, cells from 8 vials were thawed successfully inside the incubator or 37°C water bath with cell viabilities greater than 80%. Cells grew satisfactorily after thaw and exhibited a characteristic cell doubling time of approximately 30 hours during the exponential growth phase. When infected with Ad5CMV-p53 virus in the late log growth phase, a cell-specific productivity of 88000 vp/cell was attained. Similar virus productivity was maintained up to a cell passage number

of 55 (15 more cell generations) after thaw as documented in **QR04017** *ADVEXIN End of Production Cell Evaluation (C/N05498001/C/N001263).*

Cells from one of the 9 vials recovered failed to grow after thaw. The lack of cell growth was attributed mainly to the low amount of total viable cells and low cell viability observed in this vial. To prevent future lack of cell growth caused by variations in total viable cells in vials from this WCB, two vials are recommend for thaw and the thawed cells will be seeded at a target seeding density of $3.5x10^4$ cells/cm^2.

In summary, the 293 WCB P/N 08-00005, C/N001263 demonstrated acceptable functionality qualities and is recommended be released for manufacturing use upon successful completion of other quality control tests.

References

LT004-01 Cell enumeration and viability assessment
BR089-10 and 11 Cell culture expansion to see the CellCube 4x100